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Clinical Significance of p53, nm23, and bcl-2 in $T_{3-4}N_1M_0$ Oesophageal Carcinoma: An Immunohistochemical Approach

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Background and Objective: The purpose of this retrospective study was to test whether the expression of p53, nm23, and bcl-2 in $T_{3-4}N_1M_0$ oesophageal carcinoma is associated with patient survival.
Methods: Immunohistochemical localisation of p53, nm23, and bcl-2 was performed on formalin-fixed, paraffin-embedded tissue sections (N = 46). The observed range of follow-up period was 0.2–24.0 months with a median of 11.0 months. A total of 85% (39/46) of the patients died within 24.0 months, which could be due to advanced disease at presentation. The immunohistochemical signal was expressed as the proportion of positive cells. The immunostaining for p53 was nuclear, whereas that for nm23 and bcl-2 was cytoplasmic in the neoplastic cells.
Results: p53 was expressed in 70% (32/46) of cases; nm23 in 29% (13/45), and bcl-2 in 67% (29/43) of tumours. The univariate analysis showed that the expression of two markers, i.e., expression of p53 and absence of nm23 were independently associated with unfavourable overall survival time. Despite a small number of patients treated with adjuvant therapy, we observed that tumours positive for p53 had an unfavourable prognosis when compared with tumours negative for p53.
Conclusions: Our preliminary findings suggest: expression of p53 and nm23 negativity may be related to an unfavourable prognosis in patients with advanced oesophageal carcinoma.
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KEY WORDS: **oesophageal carcinoma; p53; nm23; bcl-2; prognosticator**

INTRODUCTION

Multimodality treatment of oesophageal carcinoma has been developed to obtain good local control and to prolong patient survival. Despite advances in multimodality therapy, the prognosis for patients with advanced carcinoma remains poor. Incorporation of molecular or cell-surface tumour markers into conventional clinicopathologic prognosticators may improve the reliability of prediction concerning tumour aggressiveness [1,2].

Carcinogenesis of the oesophageal epithelium is an extremely complex multistep process [3]. Evidence exists that aberrations of oncogenes and tumour suppressor genes are probably essential for this process. The gene mutations may produce metabolically more stable proteins, which can be used as prognostic indicators in patients with oesophageal cancer. Mutation of the p53 tu-

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mour suppressor gene is related to the transformation and progression of several human tumours [4–10]. Studies performed on patients with oesophageal cancer showed an association between overexpression of p53 and a high grade of the tumour [11].

nm23 is a putative metastasis suppressor gene that was isolated on the basis of reduced expression in murine melanoma cell lines of high metastatic potential. The nm23 gene has been shown at the level of both protein and mRNA, to have higher expression in breast and colorectal tissues of low metastatic potential than in corresponding high metastatic variants [12,13]. However, its role in metastatic oesophageal carcinomas is not clear.

The bcl-2 proto-oncogene is involved in inhibiting apoptosis. The product of this proto-oncogene is a 26kd protein that blocks apoptosis. More recently, several human tissues other than haemolymphoid cells have been shown to express bcl-2, including the regenerative basal crypt compartment of the small bowel and colon [14–16]. bcl-2 appears to be important in many continuously replaced epithelia, such as that of the oesophagus, presumably through prevention of cell death in regenerative compartments.

Therefore, the aim of this preliminary study was to test whether the expression of p53, nm23, and bcl-2 in oesophageal carcinomas is associated with patient survival. We chose to determine these marker expressions by immunohistochemistry since neoplasms are heterogeneous and contain subpopulations of cells differing in metastatic potential, and this method enables us to identify such variations.

MATERIALS AND METHODS

The oesophageal tissues were collected from patients who had undergone oesophagectomy at our institute between January 1987 and December 1992. Of the 46 patients, 10 had inoperable disease, and their biopsy samples were collected. Formalin-fixed, paraffin-embedded tissue blocks were retrieved from the files of the Department of Pathology of our institute. Disease was staged according to the UICC TNM (p) classification [17]. The histological assessments were performed independently by two pathologists according to the criteria established by the World Health Organisation (WHO). Patient and tumour characteristics and the treatment given are summarised in Table I.

The treatment offered was surgery only (28 patients), adjuvant radiotherapy (5,000 R) or chemotherapy (5-fluorouracil plus methotrexate, standard regimen; 6 courses; 8 patients), and the remaining patients, who had inoperable disease (N = 10), were treated with radiotherapy or chemotherapy. Postoperative radiotherapy or chemotherapy was commonly administered to patients who had a palliative resection. As the registered patients had advanced disease, recurrence-free survival time was not taken into consideration. Follow-up of the patients was until the time of death, or 2 years. The observed range of follow-up was 0.2–24.0 months with a median of 11.0 months. A total of 85% (39/46) of the patients died within 24 months, which could be attributed to advanced disease at presentation.

TABLE I. Oesophageal Carcinoma: Patient and Tumour Characteristics

Characteristics	No. of patients	Percentage
Total patients	46	100
Age (years)	Range 25–76	
Sex		
Male	31	67
Female	15	33
Habit		
Smoking	24	52
Smoking + alcohol consumption	2	4
Without	20	44
Site		
Lower third	35	76
Middle third	11	24
Tumour		
$T_3N_1M_0$	44	96
$T_4N_1M_0$	2	4
Histologic type		
Epidermoid carcinoma	33	72
Adenocarcinoma	10	22
Adenosquamous carcinoma	3	6
Histologic grade		
I	3	6
II	26	57
III	17	37
Immunohistochemical status		
p53 (N = 46)		
negative	14	30
positive	32	70
nm23 (N = 45)		
positive	13	29
negative	32	71
bcl-2 (N = 43)		
negative	14	33
positive	29	67
Treatment offered		
Surgery	28	61
Adjuvant radio- or chemotherapy	8	17
Inoperable treated with radio- or chemotherapy	10	22

Immunohistochemical Analysis of p53, nm23, and bcl-2

All three proteins were determined immunohistochemically on 3–5-μm-thick sections of formalin-fixed, paraffin-embedded tissue blocks. Sections were deparaffinised and treated with 3% H_2O_2 to block the endogenous peroxidase activity. The sections were saturated for free nonspecific protein binding sites with normal rabbit serum for monoclonal antibodies and normal swine serum for polyclonal antibodies diluted 1:10 in

Tris-buffered saline (TBS, 0.05 M Tris-HCl in isotonic solution, pH 7.6) for 20 minutes at room temperature.

For immunostaining of p53 (N = 46) and bcl-2 (N = 43), respectively, monoclonal mouse antihuman antibodies were diluted in TBS and incubated as follows: p53 (1:50, DO-7, DAKO, Glostrup, Denmark) for 2 hours and bcl-2 (1:40, 124, DAKO), for 30 minutes at room temperature. For nm23 (N = 45), polyclonal rabbit antibody to human nm23 protein was used (anti-nm23/nucleoside diphosphate kinase A, 1:50, Boehringer Mannheim, Mannheim, Germany) for 2 hours at room temperature.

The avidin-biotin-peroxidase complex technique was used for the localisation of these proteins. The sections were intensely washed with TBS. Sections were allowed to react with secondary antibody (supersensitive multilink biotinylated IgG, Biogenex, San Ramon, CA) followed by tertiary antibody (supersensitive peroxidase conjugated streptavidin, Biogenex), for 40 minutes each at room temperature. The specific immune reaction was revealed using 3′,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) as chromogen and 0.1% H_2O_2 as substrate in 0.05 M Tris-HCl (pH 7.6). The sections were counterstained with haematoxylin. Respective positive controls included tissue sections known to exhibit high levels of the protein. Negative controls were obtained by omission of the primary antibody.

For all the immunostaining assays, tumours were scored by assessing the site of staining (nuclear: p53, cytoplasmic: nm23 and bcl-2) and the proportion of stained cells was scored by a semiquantitative score. The percent positivity was determined and the cases were separated into four categories: < 10% positive tumour cells: negative; 10–30% positive tumour cells: +; 30–50% positive tumour cells: ++, and > 50% positive tumour cells: +++.

Statistical Analysis

The Kaplan and Meier model [18] was used for overall survival curves. Chi-square test with Yates' correction was used for data analysis [19]. Two-tailed *P* values of < 0.05 were considered significant.

RESULTS

Immunohistochemical Analysis of Markers

Positive reaction of the p53 to the DO-7 was nuclear. Altogether, 32 of 46 (70%) tumours showed nuclear immunoreactivity for the p53 protein (Table II). The positivity was highest in the poorly differentiated tumours followed by moderately and well-differentiated tumours (Table III).

Staining for nm23 and bcl-2 was cytoplasmic. Of the 45 patients, 32 (71%) patients showed negative nm23 staining and 67% (29/43) tumours were positive for bcl-2 immunostaining. However, no significant variation was observed when histologic type and differentiation of the tumour were considered (Tables II and III).

TABLE II. Immunohistochemcial Scores of p53, nm23 and bcl-2 in Patients With Oesophageal Carcinoma

	Score	No. of patients	Percentage
p53	−	14	30
	+	26	57
	++	6	13
	+++	0	0
nm23	+++	0	0
	++	1	2
	+	12	27
	–	32	71
bcl-2	−	14	33
	+	24	56
	++	4	9
	+++	1	2

TABLE III. Distribution of p53, nm23, and bcl-2 According to Histological Type and Grade of Tumour in Patients With Oesophageal Carcinoma

	No. of patients	p53+	nm23−	bcl-2+
Histologic type				
Epidermoid carcinoma	33	21/33 (64%)	20/32 (63%)	23/31 (74%)
Adenocarcinoma	10	9/10 (90%)	9/10 (90%)	6/10 (60%)
Adenosquamous carcinoma	3	2/3 (67%)	3/3 (100%)	0/3 (0%)
Histlogic grade				
I	3	1/3 (33%)	2/3 (67%)	1/2 (50%)
II	26	17/26 (65%)	18/26 (69%)	18/25 (72%)
III	17	14/17 (82%)	12/16 (75%)	10/16 (63%)

Prognostic Value of Markers

In a univariate cutpoint analysis, patients with p53 positive staining had a lower overall survival at 2 years than those with p53 negative staining. This difference was statistically significant ($\chi^2 = 3.43$, df = 1, $P < 0.0007$, Fig. 1).

A decreased overall survival was observed for patients having nm23 negative tumours when compared with those having nm23 positive tumours ($\chi^2 = 1.97$, df = 1, $P < 0.048$, Fig. 2).

No significant difference in overall survival was observed between the two subgroups, i.e., those with bcl-2 positive staining and those with bcl-2 negative staining (Fig. 3).

Prognostic Value of p53 and nm23

Bivariate analysis showed that the subset of patients with p53 negative and nm23 positive staining had the

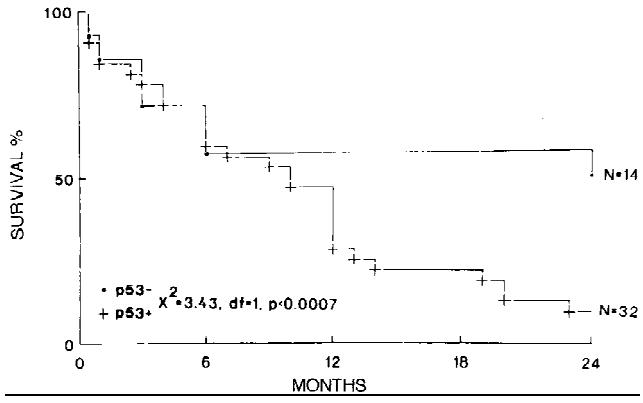

Fig. 1. Patients with p53+ tumours had significantly reduced overall survival time compared to patients with p53− tumours. (N = number of patients, − = negative, + = positive).

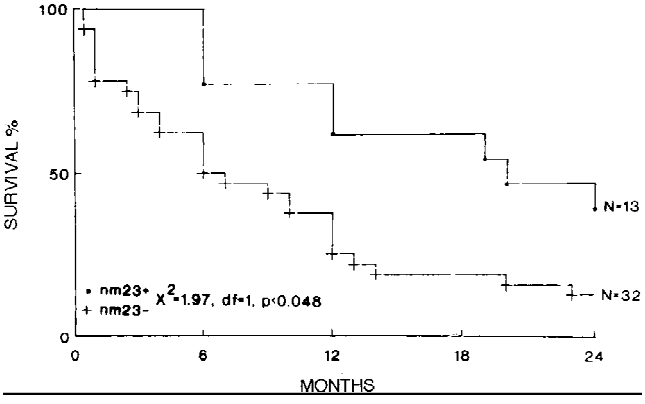

Fig. 2. Significantly reduced overall survival time in oesophageal cancer patients with nm23− tumours compared to those with nm23+ tumours. (N = number of patients, − = negative, + = positive).

best prognosis. Patients with p53 expression and nm23 negativity had the worst prognosis followed by the p53 and nm23 positive, and p53 and nm23 negative groups. Patients with p53 negative and nm23 positive tumours had a significantly better overall survival time than those with p53 positive and nm23 negative tumours (χ^2 = 4.00, df = 1, $P < 0.00006$) and those with p53 and nm23 positive tumours (χ^2 = 2.43, df = 1, $P < 0.015$, Fig. 4).

Results of Adjuvant Therapy and Clinical Usefulness of Markers

To determine the treatment benefits, 36 patients were divided into two groups: (1) patients treated with surgery only (N = 28) and (2) patients treated with adjuvant radiotherapy or chemotherapy (N = 8). For patients treated with adjuvant therapy when grouped for prognostic value of the markers, overall survival was statistically significant only between patients with p53 negative and p53 positive immunostaining (χ^2 = 2.82, df = 1, $P <$ 0.005, Fig. 5). In patients treated with surgery, there was a trend toward better overall survival in patients with p53

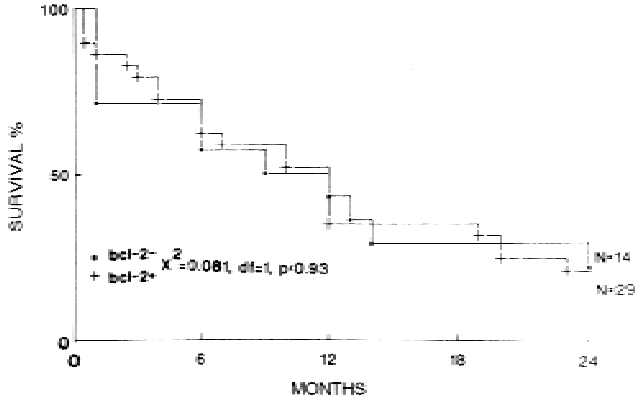

Fig. 3. Survival curves of oesophageal carcinoma patients with bcl-2− versus bcl-2+ tumours. The difference between the curves was not statistically significant. (N = number of patients, − = negative, + = positive).

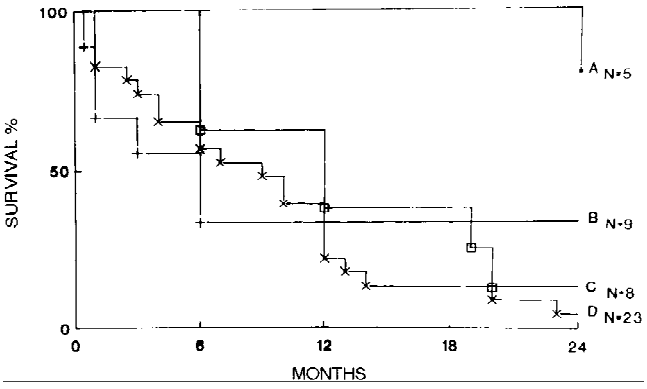

Fig. 4. Groups (**A** = p53− and nm23+, **B** = p53− and nm23−, **C** = p53+ and nm23+, **D** = p53+ and nm23−) depicting overall survival period in patients stratified by p53 and nm23 expression. Patients with p53− and nm23+ (A) immunostaining had favourable overall survival when compared with p53+ and nm23− (D, χ^2 = 4.00, df = 1, $P <$ 0.00006) and p53+ and nm23+ (C, χ^2 = 2.43, df = 1, $P < 0.015$; N = number of patients, − = negative, + = positive).

negative immunostaining than in their counterparts (Fig. 5). In contrast, in these patients, there was no difference in overall survival between nm23 and bcl-2 positive and negative groups.

The median overall survival for patients with inoperable disease treated with radiotherapy or chemotherapy (N = 10) was 6 months (range = 0.25–24 months, M ± SE = 7.24 ± 2.02), and 90% of the patients died within 12 months.

DISCUSSION

In the current study, p53 immunostaining was found in 70% (32/46) of the oesophageal carcinomas. We chose the immunohistochemical localisation technique because it is relatively rapid, simple, and results are consistent and can be performed on a day-to-day basis. On the contrary, its most evident limitation is the difficulty in

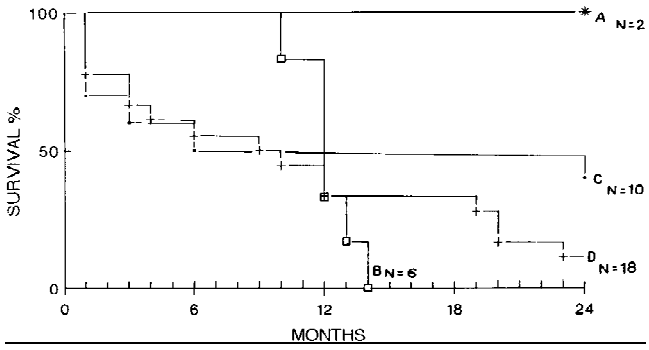

Fig. 5. Overall survival period of patients treated with surgery and adjuvant therapy. Four groups indicate (**A**) patients treated with adjuvant therapy and had p53− tumours, (**B**) patients treated with adjuvant therapy and had p53+ tumours (A vs. B: $\chi^2 = 2.82$, df = 1, $P < 0.005$); (**C**) patients treated with surgery and had p53− tumours and (**D**) patients treated with surgery and had p53+ tumours (C vs. D: $\chi^2 = 1.78$, df = 1, $P < 0.078$; N = number of patients, − = negative, + = positive).

reproducing the evaluation among different pathologists. We found p53 expression in 82% of poorly differentiated tumours. A strong relationship between p53 expression and tumour cell differentiation was observed by Lam et al. [11] and Sarbia et al. [20] using DO-7 and DO-1 antibodies, respectively, in patients with oesophageal cancers. Our preliminary results suggest that quantitative information on the accumulation of nuclear p53 correlated well with the prognosis of patients with advanced oesophageal cancer. This suggests the possibility that p53 alterations occur more often as a late event in the transformation or are associated with increased metastatic potential. A consistent observation is now emerging correlating the loss of p53 function with shortened survival in patients with cancer of the oesophagus, oropharynx, colon, lung, bladder, prostate, and breast, as well as soft tissue sarcomas and myelogenous leukemia [4–10]. Despite a small number of patients treated with adjuvant therapy, we observed that tumours positive for p53 had an unfavourable prognosis when compared with tumours negative for p53. This finding at present is being investigated in a larger patient series.

Metastasis is a multistep process and if nm23 has antimetastatic properties, it is not clear at which step it acts. Activation of the nm23 gene might be a prerequisite for oncogenesis in oesophageal carcinoma, whereas an alternate possibility is the modification of cellular characteristics in relation to proliferation and/or differentiation as a consequence of oncogenesis [13]. We have found 71% nm23 negativity in patients with oesophageal carcinoma. It may be that higher nm23 negativity expression in this situation is a marker of metastatic potential. Results from our study have shown that oesophageal carcinoma patients with nm23 negativity had significantly reduced overall survival as compared to those with nm23 positive tumours.

Recently, emphasis has been placed on the role of apoptosis and its regulation in tissue homeostasis and carcinogenesis. In the oesophagus, apoptosis has been demonstrated to play an active role in the maintenance of the mucosa. In the gastrointestinal tract, bcl-2 protein has been detected immunohistochemically in the proliferating zone, where it reputedly protects stem cells from apoptosis [21]. bcl-2 protein expression, observed in 67% of the cases, appears to be a frequent event in oesophageal epithelial neoplasms. With regard to overall survival, we failed to demonstrate any association with bcl-2 expression.

Our preliminary results suggest that expression of p53 and nm23 negativity may be indicative of unfavourable prognosis in patients with advanced oesophageal carcinoma. Analysis of larger cohorts of oesophageal cancer are needed to examine the wider applicability of our findings.

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